BELSPO Postdoctoral fellowship for 18 months: Dr. Vitalina Gryshkova

Project title: Investigation of Thrombopoietin Receptor Dimerization and Signaling Mechanisms

Final report

Dimerization and signaling of thrombopoietin receptor (TpoR).

Thrombopoietin receptor (TpoR) belongs to the single chain cytokine receptor subfamily, which also includes receptors for growth hormone (GH), prolactin (PRL), erythropoietin (EPO), granulocyte-colony-stimulating factor (GCSFR). The constitutively active mutants of TpoR (TpoRW515K, TpoRS505N) are involved in the development of myeloproliferative neoplasms (MPN), which makes them attractive targets for the investigation. The precise mechanisms of cytokine receptor activation in physiological and pathological conditions are not fully understood. Certain cytokine receptors, like EpoR or GHR, exist as pre-formed dimers on the cell surface and activation with the ligand leads to the conformational changes in the preformed dimers. However, for TpoR these aspects are not clear.

One of the aims of this project was to study the dimerization of TpoR upon ligand activation as well as the dimerization of its constitutively active mutants TpoRW515K and TpoR S505N by *Gaussia princeps* split-luciferase assay. In this assay two proteins of interest are fused to complementary fragments of *Gaussia princeps* luciferase. In case of protein interaction luciferase fragments are brought together and folded into native structure which allows the luciferase to oxidize the substrate coelenterazine (CTZ) (Fig.1).

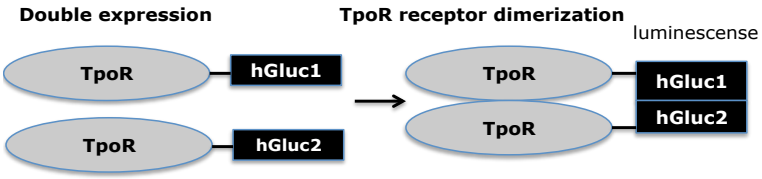

Fig.1. The scheme of the Gaussia princeps luciferase assay to study the dimerization of TpoR.

To achieve the goals set for this project I first cloned the cDNAs of murine and human TpoR into pcDNA3.1 vector containing cDNA of different fragments of *Gaussia princeps* luciferase. The expression of HA-tagged TpoR fused in frame to the luciferase at the COOH-end was confirmed by Western blot. The W515K and S505N activating point mutations were introduced in human TpoR by site-directed mutagenesis. Aforementioned DNA constructs were used to transiently transfect HEK293 cells. Split-luciferase assay was performed on live cells treated or not with TPO, as well as in cells lysates. The results showed that *Gaussia*

luciferase complementation (dimerization) of constitutively active mutants TpoRW515K and TpoRS505N was significantly higher than that of wild type TpoR either in live cells or in cell lysates. At the same time the dimerization of TpoR was significantly lower compared to that of EpoR, which exists as preformed dimer. Incubation with the ligands did not lead to the dramatic changes in receptor dimerization suggesting that these cytokine receptors are already weak preformed dimers (at least at the levels of expression in 293 cells) and ligand binding does not lead to the dimerization of monomeric forms of receptors, but rather to stabilization of an active interface that allows for conformational changes in the intracellular parts of the receptors in the preformed dimers (Fig. 2A, 2B).

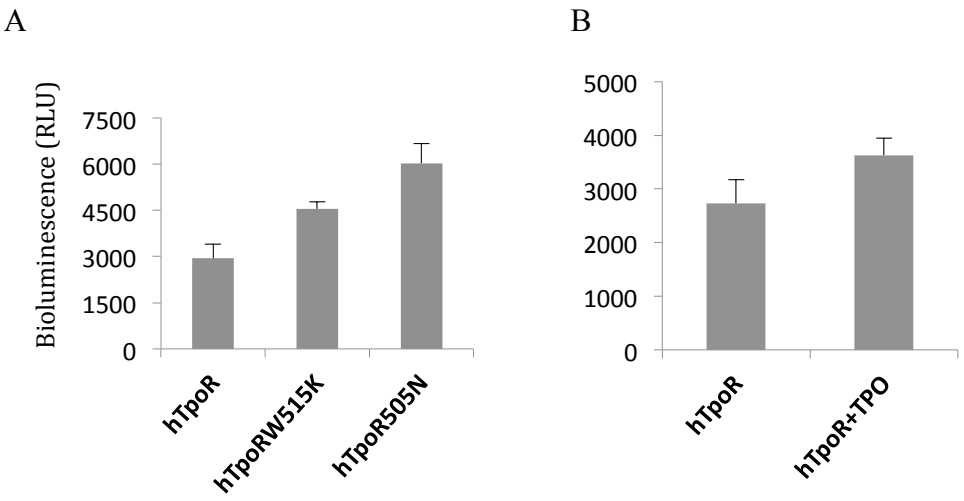

Fig. 2. A. Dimerization of TpoR constitutively active mutants TpoR W515K and S505N on live cells. B. Dimerization of TpoRwt upon prolonged incubation with TPO (50 ng/ml).

Dimerization of the wild type TpoR was compared for human and murine receptors. The assay performed either in cell lysates or in live cells did not reveal significant differences in dimerization of human full-length TpoR compared to murine one. Interestingly, the dimerization of murine TpoR lacking the whole extracellular domain (490-633 aa) led to a very high increase in dimerization (Fig. 3A) indicating that the extracellular part of the receptor could strong constitutive dimerization and constitutive activation of the receptor. Surprisingly, the same increase in complementation was not seen for human TpoR lacking extracellular domain (Fig. 3B). These data are in agreement with data we obtained by NMR in collaboration with prof. Steven O. Smith who showed the transmembrane sequence of the

murine TpoR, flanked by juxtamembrane residues is a strong dimer, while the same sequence of human TpoR is a much weaker dimer.

Dimerization induced by transmembrane and cytosolic regions of TpoR. Previously in our laboratory a series of constructs was obtained where the extracellular part of TpoR was replaced by a dimeric coiled-coil. Seven possible orientation of TpoR were obtained by fusing the Put3 dimeric coiled-coil with the transmembrane domain of the TpoR at seven different junction points. It was shown that TpoR could signal from 6 out of 7 possible conformations. To assess the dimeric state of these chimeric proteins, I engineered two active conformations, namely ccTpoR-IV and ccTpoR-0, and inactive conformation ccTpoR-II, as fusion proteins at their COOH-terminus with the Gluc1 and Gluc2 complementing *Gaussia luciferase* fragments.

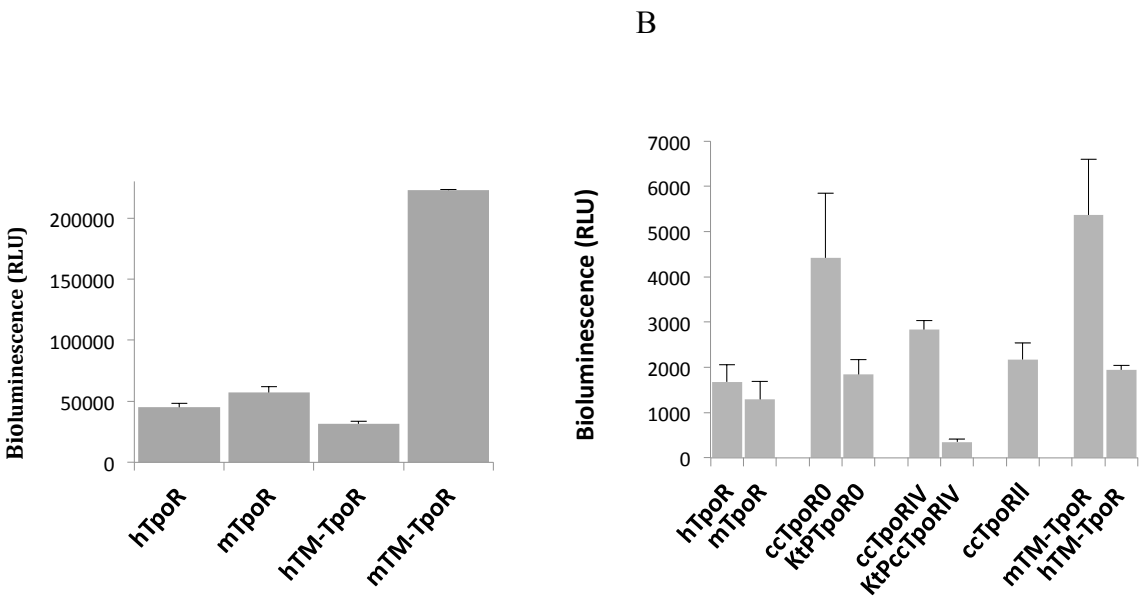

Fig.3. Dimerization of full-length TpoR, truncated forms lacking extracellular domain and coiled-coil fusions in lysates (A) and live cells (B).

A mutation – K86P, which disrupts dimerization of the coiled-coil, was introduced in the constructs made for split-luciferase assay. In these constructs dimerization can only be caused by transmembrane and/or cytosolic domains of TpoR. The results of dimerization showed that ccTpoR-0 and ccTpoR-lV are much stronger dimers than the wild type murine TpoR, which confirm that notion that the extracellular domain prevents dimerization, as

detected in Fig. 3A. Disruption of the coiled-coil by K86P exerted different effects on ccTpoR-IV versus ccTpoR-0. Namely the K86P mutation inhibited, but not completely, dimerization of ccTpoR-lV, but it did not affect dimerization of ccTpoR-0. This would indicate that the TM-cytosolic sequences promote dimerization in the register of dimerization of the ccTpoR-0, as opposed to the register of ccTpoR-IV, where the TMs cannot dimerize strongly. Molecular dynamics simulations are on-going to establish the exact energy contributions for these interfaces. We also compared the dimerization levels of ccTpoR-0, ccTpoR-IV, which are active dimers, and ccTpoR-II, which represents the inactive dimer, and is a model for the inactive conformation of TpoR. As depicted in Fig.3, ccTpoR-II and cc-TpoR-IV exhibit similar dimerization levels, but ccTpoR-0 exhibits higher levels of complementation/dimerization. Only when the coiled-coil is disrupted by the K86P mutation do levels of dimerization of ccTpoR-0 decrease to the levels of ccTpoR-II and IV. Taken together these data show that the conformation of ccTpoR-0 is stabilized by the dimerization of the transmembrane/cytosolic domains when the register imposed is that of fusing the Put3 to residue T484. This dimerization decreases when the register is changed by fusing to L486 (two residues down) or to residue T488 (four residues down). Either the rotations (220 or 440 degrees counter clockwise) or the absence of the first two I484-T485, or first 4 transmembrane residues (I484-T485-L486-V487) could explain the decrease in TM-cytosolic dimerization.

Dimerization and signaling by JAK2 and JAK2V617F and interaction between JAK2 or JAK2V617F with TpoR.

At the next stage the dimerization of JAK2 kinase, which is the main kinase providing downstream signalling for TpoR, was studied in detail. The COOH-termini of JAK2 or of the MPN-associated mutant JAK2V617F, were fused to complementary fragments of *Gaussia princeps* luciferase and transiently expressed in HEK293 cells. The dimerization of JAK2 or JAK2V617F was assessed upon co-expression with thrombopoietin receptor in the absence or presence of the ligand by measuring luciferase signal in cell lysates. Prolonged incubation with the TPO (>3 h at 30-37° C) led to significantly higher dimerization of JAK2 suggesting that the COOH-ends of JAK2 arrive in close proximity for activation, leading to trans-phosphorylation and initiation of signalling. Furthermore, *Gaussia* complementation between JAK2V617F Gluc1 and Gluc2 fusion proteins was significantly higher than that of the wild type JAK2, suggesting constitutive close proximity of the COOH-termini of the kinase domains for JAK2V617F.

It is known that cytokine receptors like the TpoR, the EpoR, or the GCSFR were shown to support JAK2V617F constitutive signaling in hematopoietic cells, presumably via their tendency to homodimerize in the absence of ligands. Co-expression with TpoR further increased the complementation signal for JAK2V617F compared to wild type JAK2, which correlated with the levels of phosphorylation of the kinase at activation loop Y1007 (Fig. 4).

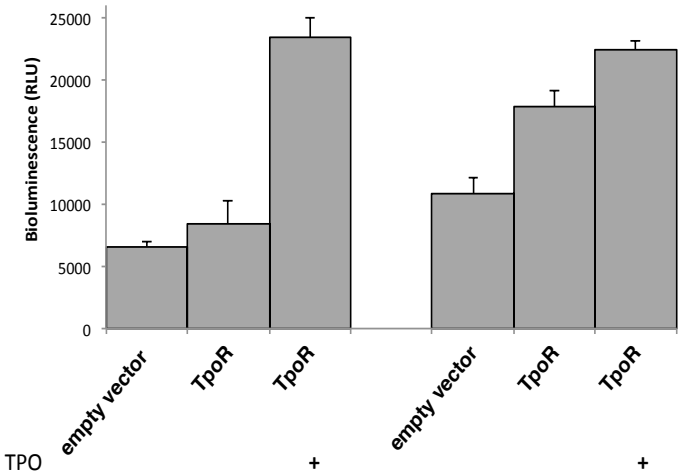

Fig. 4. Complementation of JAK2 V617F in presence of different cytokine receptors

Moreover, I used this system to assess the dimerization of JAK2V617F in presence of different dimeric cytokine receptors that normally utilize JAK2 in the myeloid lineage (EpoR, TpoR, GCSFR). The results showed that TpoR promoted further JAK2V617F dimerization over that exhibited by JAK2V617F alone (Fig. 5). That was not the case for the EpoR or GCSFR (Fig. 5).

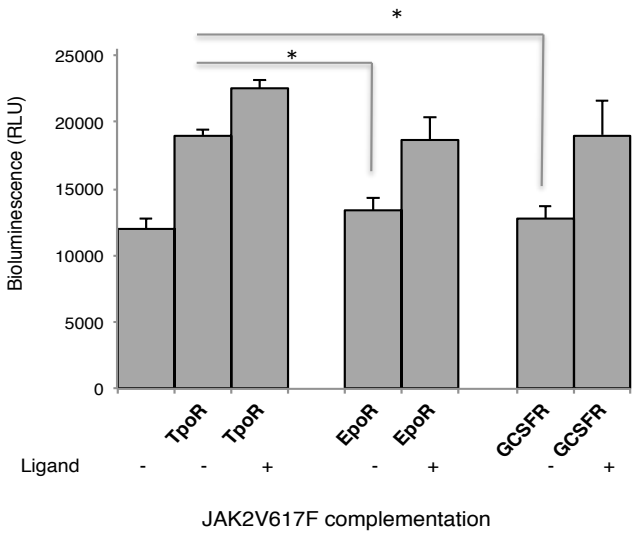

Fig. 5. Complementation of JAK2V617F fused to Gluc1 and Gluc2 in presence of different dimeric cytokine receptors.

To compare the interactions between TpoR and JAK2 versus JAK2V617F, split-luciferase assays were performed between the JAK2 or JAK2V617F and cytokine receptors, all fused at their COOH-ends with Gluc1 or Gluc2. These experiments revealed stronger interaction between TpoR and JAK2V617F when compared to TpoR and wild type JAK2 (Fig. 6). This difference could be the result of higher affinity between TpoR and JAK2V617F versus JAK2 wild type. Since the NH2-terminus FERM domain that is supposed to mediate interaction with TpoR is identical in the two JAK2 proteins, this suggests that the kinase domain activation enhances or stabilizes the interaction with TpoR. Alternatively, or in addition, the complexes might be more stable for JAK2V617F and TpoR, or the proximity between the C-terminus of TpoR and the kinase domain of JAK2 might be higher in the case of the conformation of JAK2V617F.

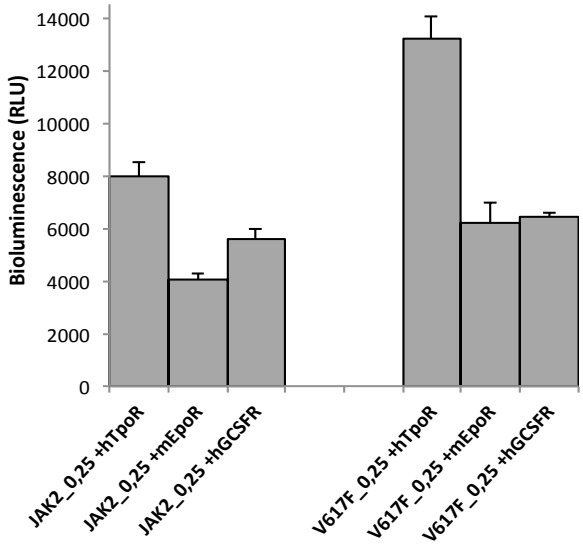

Fig. 6. Complementation between JAK2 or JAK2 V617F and cytokine receptors

Interactions of the p85 subunit of PI3K and cytokine receptors or JAK2/JAK2V617F.

To investigate the interaction between TpoR and PI3K by split luciferase assay, the regulatory subunit of PI3K - p85 was fused to *Gaussia princeps complementing* fragments Gluc1 and Gluc2 and was co-expressed with hGluc-tagged TpoR in the presence or absence of activation by Tpo. The experiments on the complementation between p85 and TpoR showed that the interaction between these two proteins was weak and was not changed after incubation with

Tpo. In contrast, p85 interacted with EpoR (not shown), confirming other publications that showed EpoR-p85 interactions by other methods. At the next step I tested the interaction between p85 and JAK2 as well as JAK2V617F. The data clearly showed that the interaction between p85 and constitutively active JAK2V617F was stronger than with wild type JAK2 (Fig. 7) suggesting that p85 could bind to phosphorylated form of JAK2. Stronger interaction between JAK2 and p85 was noticed upon co-expression with TpoR and activation with Tpo suggesting again possible involvement of phosphorylated JAK2 in this interaction. The interaction between JAK2V617F and p85 was inhibited by type 1 (ruxolitinib) and type II (BBT-594) JAK inhibitors, which act differently on JAK2 kinase. It is known that ruxolitinib could increase the phosphorylation of Y1007/1008 on JAK2, whereas BBT-594 decreases this phosphorylation dramatically. I probed the samples used for complementation with the antibodies against p-Y1007/1008 JAK2 and confirmed the phosphorylation status of JAK2 (Fig. 7). These data suggest that interaction between p85 and JAK2 probably does not depend on phosphorylation of Y1007 of JAK2. However we hypothesize that it depends on other phosphorylated tyrosines in JAK2. In contrast, inhibitors of PI3K catalytic subunit p110, like the BEZ-335 inhibitor, did not affect the interaction between JAK2V617F and p85. These data therefore establish that the interaction between p85 and JAK2V617F depends only on the catalytic activity of JAK2V617F. Further research is needed to reveal the mechanism of this interaction and this could be subject of future collaboration between Belgian and Ukrainian laboratories.

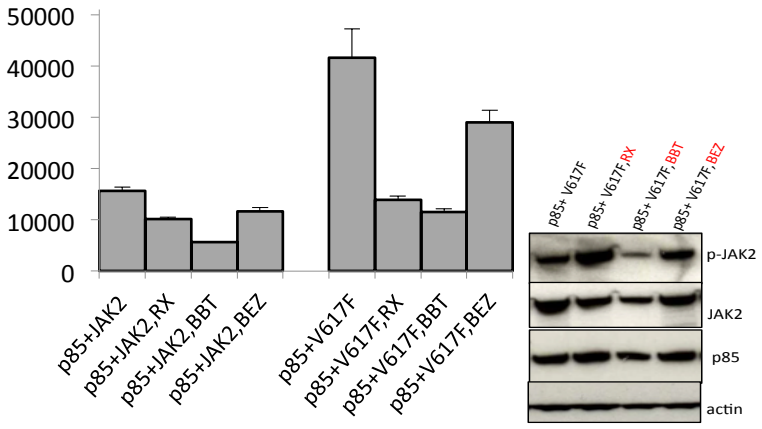

Fig. 7. The inhibition of the interaction between p85 and JAK2V617 by JAK2 inhibitors and not by PI3K inhibitors.

Publications

Vitalina Gryshkova, Salwa Najjar and Stefan N. Constantinescu The Long-Lived Thrombopoietin Receptor Supports Prolonged JAK2 V617F Dimerization and Activation when Compared to other Dimeric Cytokine Receptors. Manuscript in preparation.

Caroline Marty, Christian Pecquet, Harini Nivarthi, Ilyas Chachoua, Roxana I. Albu, Kahia Messaoudi, Ciara Cleary, Doris Chen, **Vitalina Gryshkova**, Jean-Philippe Defour, Isabelle Plo, William Vainchenker, Stefan N. Constantinescu, Robert Kralovics Myeloproliferative Neoplasm Associated Mutants of the Chaperone Calreticulin Induce Activation of Thrombopoietin Receptor and Thrombocytosis In vivo, *Submitted*, 2014

Shochat C, Tal N, **Gryshkova V**, Birger Y, Bandapalli OR, Cazzaniga G, Gershman N, Kulozik AE, Biondi A, Mansour MR, Twizere JC, Muckenthaler MU, Ben-Tal N, Constantinescu SN, Bercovich D, Izraeli S. Novel activating mutations lacking cysteine in type I cytokine receptors in acute lymphoblastic leukemia. *Blood*. **124**(1):106-110, 2014.

Constantinescu S.N., Leroy E., **Gryshkova V.**, Pecquet C. and Dusa A. Activating Janus Kinase pseudokinase domain mutations in myeloproliferative and other blood cancers. *Biochem. Soc. Trans.* **41**(4): 1052-1058, 2013.